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Naturally occurring amino acid substitutions in the HIV-2 ROD envelope glycoprotein regulate its ability to augment viral particle release

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Abstract

The envelope glycoprotein of HIV-2 ROD10 has the intriguing ability to enhance the rate of viral particle release from infected cells. However, not all HIV-2 envelope glycoproteins are active in this regard. Indeed, we have previously noted that, despite a high degree of identity with that of ROD10, the envelope protein of the ROD14 isolate was unable to enhance virus production. In this study, site-directed mutagenesis was employed to reveal that a single naturally occurring alanine-to-threonine substitution at position 598, located in the extracellular part of the TM subunit, fully accounted for the lack of activity of the ROD14 Env in HeLa and 12D7 cells. A second mutation at position 422, substituting a lysine residue in ROD10 for an arginine in ROD14, was additionally required for efficient virus release from infected H9 cells, suggesting cell-type-specific requirements for this activity. Interestingly, the ROD14 Env protein exhibited a *trans*-dominant negative effect on particle release by ROD10 Env, suggesting that the viral release activity of the HIV-2 ROD envelope protein may be regulated by its ability to assemble into functional oligomeric structures.

Introduction

Vpu is a human immunodeficiency virus type 1 (HIV-1)-specific protein that enhances the release of viral particles from infected cells (Strebel et al., 1989; Terwilliger et al., 1989). Despite the absence of a genuine *vpu* gene, some isolates of HIV-2, such as ROD10 and ST, express a functional homologue to Vpu. Indeed, the envelope glycoprotein of these viruses was shown to enhance the rate of viral particle production in a manner indistinguishable from that of genuine Vpu (Bour et al., 1996; Ritter et al., 1996). Both Vpu and the ROD10 Env augment the release of chimeric viruses bearing the *gag-pol* regions of heterologous retroviruses, including HIV-1, HIV-2, and simian immunodeficiency virus (SIV) (Bour and Strebel, 1996; Göttlinger et al., 1993), suggesting a common mechanism of action between these two proteins in enhancing particle release. However, the precise mechanism by which Vpu enhances virus release has not been fully uncovered despite recent data suggesting that it may involve the formation of an ion channel by oligomeric Vpu (Ewart et al., 1996; Grice et al., 1997; Schubert et al., 1996b). One intriguing possibility is that the HIV-2 Env could similarly mediate the release of viral particles through the formation of a membrane pore. This is supported by the fact that Vpu and the HIV-2 Env both require the presence of a functional *trans*-membrane domain for their activity (Bour et al., 1996; Schubert et al., 1996a) and adopt an oligomeric structure favorable to the formation of a membrane pore (Maldarelli et al., 1993; Rey et al., 1990).

Expression of the *vpu* gene is regulated at the translational level. Indeed, a number of HIV-1 isolates that harbor a functional *vpu* coding sequence do not encode the Vpu protein due to the absence of a functional initiation codon (Korber et al., 1997). Although the nature of the selection pressure driving the elimination of Vpu is presently not

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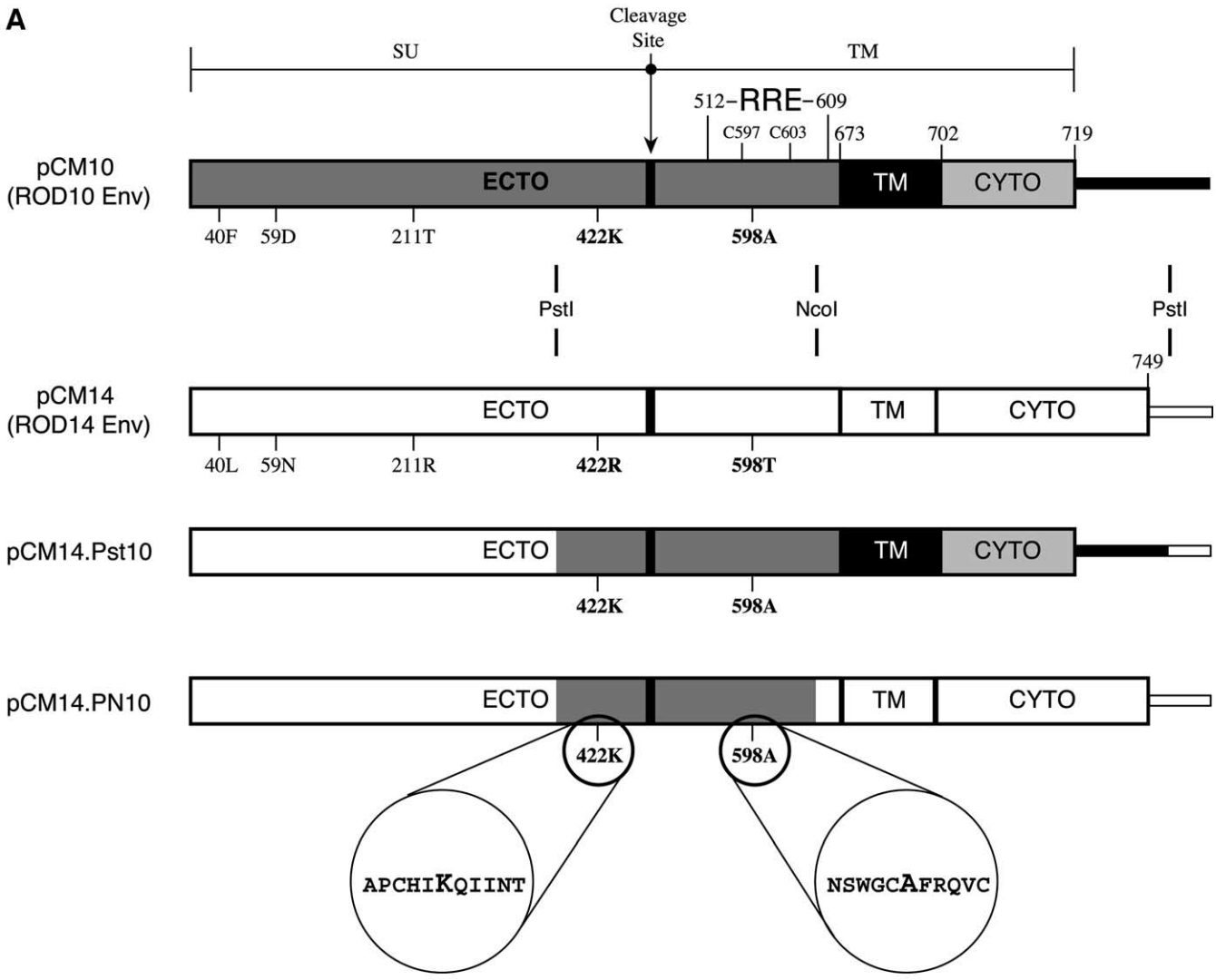
clear, the resulting decrease in viral particle production could contribute to the establishment of long-lasting latent infections. Previous studies have suggested that HIV-2 also has the ability to regulate the particle release activity of its envelope protein by modifying the length of the transmembrane (TM) subunit cytoplasmic tail (Ritter et al., 1996). However, such putative correlation between the length of the cytoplasmic tail and the presence of particle release-promoting activity could not be confirmed in the case of the ROD10 isolate (Bour et al., 1999b). The present work aims at resolving this apparent contradiction by defining the determinants in the ROD10 envelope protein that regulate its particle release activity. To this end, we examined the particle release activity of ROD14, a molecular clone of HIV-2 closely related to ROD10 that originated from the same patient (Clavel et al., 1986; Naidu et al., 1988; Ryan-Graham and Peden, 1995). We demonstrate that despite its high level of homology with the active ROD10 envelope, the ROD14 envelope does not support viral particle release. Site-directed mutagenesis performed on the ROD14 as well as the ROD10 envelope shows that the ability of HIV-2 ROD to enhance viral particle release is not regulated by the length of the cytoplasmic tail but by two amino acid substitutions in the ectodomain of the envelope glycoprotein.

Results

Delineation of a functional region for particle release activity within the extracellular domain of Env

We have previously reported that the envelope protein of ROD14, an HIV-2 molecular clone closely related to ROD10, was unable to enhance viral particle release (Bour et al., 1999b). We have also shown that this activity is not regulated by the length of the Env protein cytoplasmic tail (Bour et al., 1999b). To define the regions in HIV-2 Env important for viral particle release, chimeras were constructed between the ROD10 and ROD14 envelope genes and cloned under the transcriptional control of the CMV promoter (pCM10 and pCM14, respectively; Fig. 1A). The pCM14.Pst10 chimera contains the *Pst*I fragment from the ROD10 Env and introduces amino acid changes 422RK and 598TA as well as a premature stop codon that truncates the cytoplasmic domain to 18 residues (Fig. 1A). Construct pCM14.PN10 is similar to pCM14.Pst10 but in the context of the 48-residue ROD14 Env cytoplasmic tail. We previously showed that ROD10.env1, an Env-deficient mutant of HIV-2 ROD10, had low particle release efficiency but could be rescued by providing in trans an envelope protein active for particle release (Bour et al., 1996). The chimeric envelope proteins were expressed in HeLa cells and assessed in pulse-chase experiments for their ability to restore in trans efficient particle release by ROD10.env1. Expression of the CMV-driven envelope genes was Rev-dependent in this context and therefore required the presence of ROD10.env1 in the same cell. As shown in Fig. 1B, the ROD14 as well as the chimeric envelopes were properly expressed and present in similar amounts in all samples. The presence of different Env variants had no apparent effect on the synthesis or maturation of Gag proteins detected on the gels. However, the presence of the CMV-driven plasmids expressing the ROD14.Pst10 and ROD14.PN10 envelopes generated a redistribution of Gag proteins from the cell to the virion fraction, indicating that these envelope species were able to rescue the particle release defect of ROD10.env1 (Fig. 1B). No such effect was observed in the presence of the ROD14 envelope (Fig. 1B). To quantify the ability of the different envelope species to promote viral particle release, the radioactive intensity of the bands corresponding to the p58gag, p26/27 CA, and p16 MA was measured and the ratio of virion-associated Gag proteins to the total combined cellular and viral Gag proteins was calculated and plotted as a function of time (Fig. 1C). As previously reported, particle release efficiency by the ROD14 molecular clone was low and similar to that observed for an Env-deficient mutant of ROD10 (Bour et al., 1999b). Transfer of a *Pst*I fragment from the active ROD10 envelope protein restored particle release activity to the ROD14 envelope (Fig. 1C, ROD14.Pst10). Furthermore, particle release efficiency of the ROD14.PN10 chimeric Env was very similar to that of ROD14.Pst10. Since the two chimeras differ only by the length of their cytoplasmic tail, these data further confirm our previous finding that the length of the HIV-2 ROD Env cytoplasmic domain does not

Fig. 1. Delineation of the HIV-2 ROD Env residues involved in regulating viral particle release. (A) Schematic representation of the CMV promoter-driven ROD10/ROD14 Env chimeric constructs. DNA fragments obtained by restriction digest of the ROD10 Env-expression vector pCM10 with *Pst*I were introduced into the ROD14 Env pCM14 vector to give rise to pCM14.Pst10. The pCM14.PN10 chimera was obtained by replacing the *Nco*I fragment in pCM14.Pst10 with that of pCM14. The amino acid sequences surrounding positions 422 and 598 are indicated on the bottom. (B) HeLa cells were transfected with 20 μg of the Env-deficient pROD10.env1 molecular clone in the presence of 10 μg of either pCM14 (ROD14), pCM14.Pst10 (ROD14.Pst10), or pCM14.PN10 (ROD14.PN10). The ability of the chimeric envelopes to enhance particle release by ROD10.env1 was assessed by pulse-chase analysis. Cells were pulse-labeled for 30 min with Trans^{35}S-Label (1 mCi/ml) and chased for the indicated times. At each time point, aliquots of the cells and the virus-containing supernatants were harvested, and virus particles present in the culture medium were pelleted. Viral proteins were recovered by immunoprecipitation of both the cell lysate and the pelleted virus fractions with a pool of anti-HIV-2 patient sera, separated on 12% SDS–PAGE, and visualized by fluorography. The HIV-2 major Gag proteins p58^{gag}, p26–27^{CA}, and p16^{MA} as well as the HIV-2 envelope glycoprotein precursor gp140 are identified on the left. (C) Bands corresponding to the HIV-2 major Gag proteins in (B) were quantified with an image analyzer. The efficiency of particle release at each time point was calculated by dividing the amount of Gag proteins present in the pelleted virus fraction by the total of cell- and virus-associated Gag proteins. The ratio of virion-associated versus total Gag protein was then plotted as a function of time.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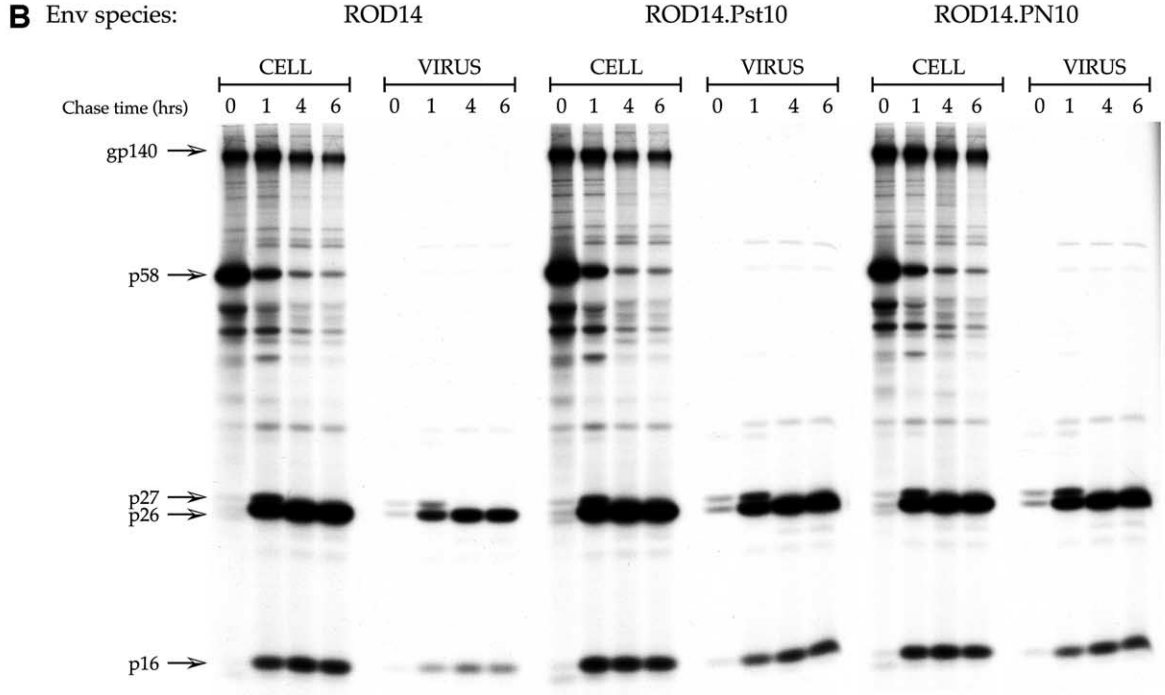
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Chase time (hrs)
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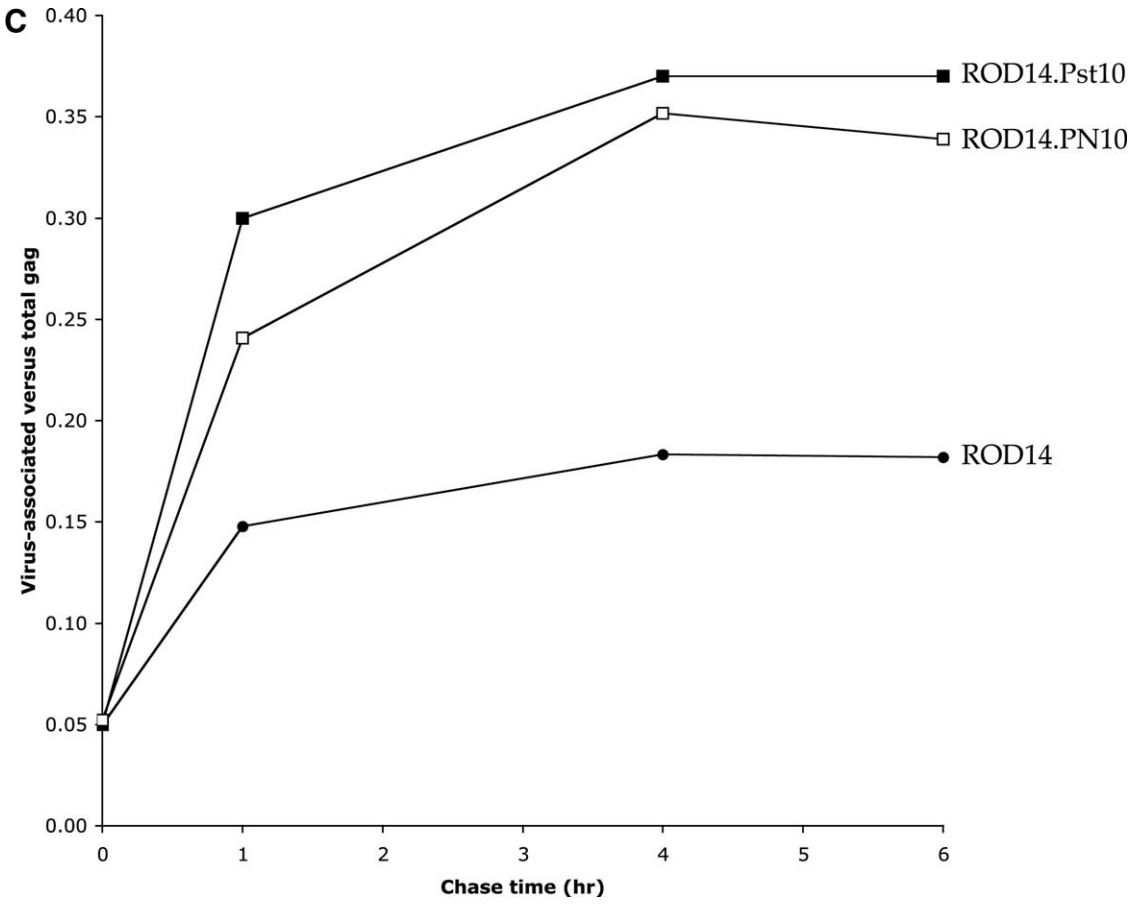

Fig. 1 (*continued*)

influence viral particle release (Bour et al., 1999b). These results indicate that the particle release activity of ROD Env is indeed regulated and that the sequences important for this regulation are comprised within the PN10 fragment.

A naturally occurring substitution at position 598 regulates the envelope activity on viral particle release in HeLa cells

The ROD10 and ROD14 envelope sequences encoded by the PN10 fragment differ by only two amino acids: a lysine-to-arginine substitution at position 422 and an alanine-to-threonine substitution at position 598 (Fig. 1A). To directly address the role of these residues in the regulation of the envelope's particle release activity, the residues at positions 422 and 598 in ROD14 were changed, either singly or in combination, to those present in ROD10. The mutations were introduced into the pROD1014 molecular clone, which carries the ROD14 *env* gene in the backbone of ROD10, and their effect on particle release was assessed by pulse-chase analysis and immunoprecipitation as described above (gels not shown). Quantification of the particle release efficiency of the ROD1014.422RK, ROD1014.598TA, and ROD1014.RK/TA double mutant showed that the 422RK mutation had little effect on the activity of the ROD14 envelope (Fig. 2, compare ROD1014 and ROD1014.422RK). In contrast, the 598TA mutation alone led to a nearly three-fold increase in particle release (Fig. 2, ROD1014.598TA). Such virus release efficiency is very similar to that observed for the wild-type ROD10 isolate (Bour et al., 1999b). These data indicate that the residue at position 598 is critical for regulating the envelope's Vpu-like activity. The presence of the 422RK mutation in addition to 598TA did not significantly improve the particle release activity from HeLa cells, in agreement with data obtained with the 422RK single mutant (Fig. 2, ROD1014.RK/TA). The 598TA mutation is therefore necessary and sufficient to restore particle release activity in the inactive ROD14 envelope in transfected HeLa cells.

Cell-type-dependent requirement for the 422RK/598TA double mutation for efficient particle release

Both the HIV-1 and the HIV-2 envelope proteins have the ability to form intracellular complexes with the CD4 receptor that can result in the blockage of both CD4 and Env in the endoplasmic reticulum (Bour et al., 1991, 1995). Since we have previously shown that the ROD10 Env is

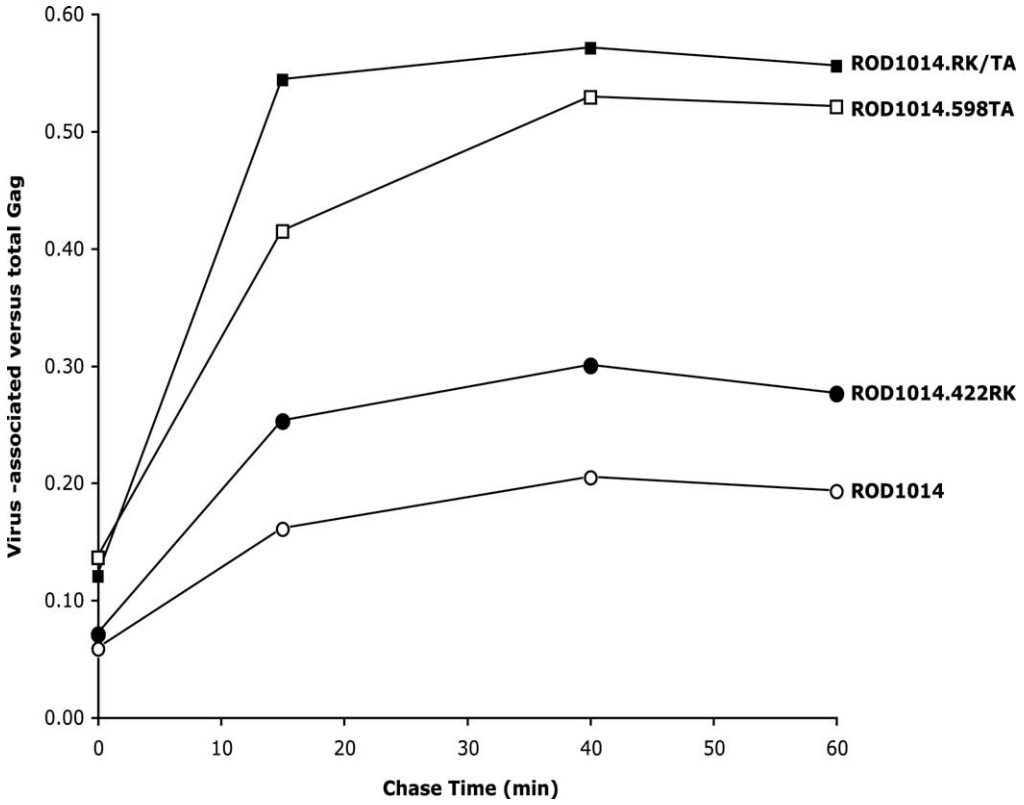

Fig. 2. The effect of the 422RK and 598TA mutations on ROD14 particle release was assessed by pulse chase after transfection of HeLa cells with 30 μg of the indicated molecular clone DNAs. Pulse-chase analysis, immunoprecipitation, and gel electrophoresis were performed as described in Fig. 1 (not shown). Gag proteins detected on the gels were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legends to Fig. 1 and the ratio of virion-associated versus total Gag proteins was plotted as a function of time.

unable to enhance particle release when trapped in the ER (Bour et al., 1996), we addressed whether the ability of the 598TA mutation to restore particle release activity to the ROD14 envelope in HeLa cells could also be observed during the course of a productive infection in $CD4^+$ cells. To guarantee equal initial infectivity of all preparations regardless of Env mutations, viruses were pseudotyped with the vesicular stomatitis virus G protein (VSV-G). To verify that the VSV-G envelope-pseudotyped viruses behaved similarly to their wild-type counterparts with regard to particle release, HeLa cells were infected with ROD1014/VSV and ROD1014.598TA/VSV and a pulse-chase experiment was performed. As shown in the top of Fig. 3A, the presence of the 598TA mutation enhanced the rate of export of virus-associated p24. Quantitation of the viral Gag proteins revealed a remarkable fourfold enhancement of viral particle release by the ROD1014.598TA mutant as compared to the original ROD1014 (Fig. 3A, bottom). This result is consistent with data presented in Fig. 2 and shows that VSV-G pseudotyping of the ROD1014 virus does not alter its particle release efficiency.

VSV-G-pseudotyped virus preparations were subsequently used to infect $CD4^+$ 12D7 and H9 cells. Cells were infected with equal amounts of ROD1014, ROD1014.422RK, ROD1014.598TA, and ROD1014.RK/TA virus stocks for 4 h at 37°C. The cells were then washed to remove residual input virus and virus replication was monitored by determining the reverse transcriptase (RT) activity in the culture supernatants harvested at 2-day intervals (Fig. 3B). In 12D7 cells, peak RT production occurred at 8 days postinfection for all viruses tested; however, the 598TA and RK/TA mutants produced over twofold higher RT activity at peak virus infection than ROD1014 and the 422RK mutant. This result is typical of the situation observed for the HIV-1 Vpu protein (Strebel et al., 1988; Yao et al., 1992) and indicates that the 598TA mutation led to an activation of the Vpu-like particle release enhancement activity of the HIV-2 ROD Env. This closely parallels the situation observed in HeLa cells and shows that, even in the presence of CD4 in 12D7 cells, the residue at position 598 in the ROD envelope protein is sufficient to enhance viral particle release. Interestingly, in H9 cells, mutation of residue 598 alone (pROD1014.598TA) was not sufficient to restore efficient virus replication. Instead, both the 598TA and the 422RK mutations were required to achieve high levels of viral particle production (Fig. 3B, pROD1014.RK/TA). These results demonstrate a functional codependence between residues 422 and 598 in the HIV-2 ROD envelope protein for regulation of virus release from certain cell types and might explain why these two mutations have coevolved.

Proteolytic cleavage is required to activate the ROD10 Env particle release activity

To better define the molecular mechanism by which residues 422 and 598 regulate the particle release activity of the HIV-2 ROD Env, we performed a comparative biochemical characterization of the wild-type ROD10 and the three ROD14 envelope mutants described above. All analyses were performed with full-length cytoplasmic tail envelope proteins. No significant differences were observed at the level of protein synthesis, stability, cell-surface expression, or virion incorporation (data not shown). This suggested that the inability of the ROD14 Env to enhance particle release was not the result of a gross modification of the envelope's structure. Since the mature TM subunit of HIV-2 Env has structural and topological features similar to that of the HIV-1 Vpu protein, we examined whether the Vpu-like activity of the ROD10 Env required proteolytic cleavage of the gp140 precursor and release of the mature gp36 TM subunit. If so, then subtle differences in the relative precursor processing efficiencies could account for the observed effects. To address this question, HeLa cells were transfected with either wild-type pROD10 plasmid DNA or an Env cleavage-deficient variant (pROD10.Clv) obtained by substituting a threonine for an arginine at position 511 to inactivate the envelope cleavage site, as previously described (Freed and Myers, 1992). A similar cleavage mutant in the context of the full-length cytoplasmic tail Env (pROD10FL.Clv) was also expressed in HeLa cells. Pulse-chase labeling and immunoprecipitations were performed as described above and viral Gag proteins were visualized and quantified as shown in Fig. 4A. The envelope cleavage site mutation had no apparent effect on the efficiency and kinetics of Gag processing, as demonstrated by the appearance of the p26/27 mature capsid products in all samples (Fig. 4A, insets). Quantification of the particle release efficiency demonstrated efficient particle release for wild-type ROD10 with 40% of the total Gag proteins found in the form of secreted viral particles within 60 min of chase (Fig. 4A, ROD10). In contrast, particle release efficiencies for both the truncated and the full-length versions of the cleavage-deficient envelope protein were significantly reduced and similar to that observed for the inactive ROD14 Env (Fig. 4A, ROD10.Clv, ROD10-FL.Clv). These results demonstrate that proteolytic cleavage of the Env precursor is required to activate its particle release activity.

We next examined whether the lack of activity of the ROD14 envelope protein could be due to inefficient precursor cleavage. To ensure quantitative detection of the mature gp36 product, a FLAG epitope tag was added to the C-terminus of full-length ROD10 and ROD14 envelope proteins in the context of the pROD-A1 subgenomic construct. Plasmids pROD-A2.flg, pROD-A14FL.flg, as well as the cleavage mutant pROD-A2Clv.flg construct were transfected into HeLa cells. Thirty-six hours posttransfection, cells were lysed in detergent buffer and equal amounts of protein extracts were subjected to SDS–PAGE. Proteins were transferred to PVDF membranes and probed in Western blot with anti-FLAG M2 antibodies. As shown in Fig. 4B, all three Env species were expressed at similar rates, as judged by the intensity of the gp140 precursor band. As expected, no mature gp36 product was observed in the case of the 511RT cleavage mutant (Fig. 4B, ROD10FL.Clv). In contrast, efficient cleavage of both the ROD10 and the ROD14 envelope proteins was observed, yielding the mature gp36 TM subunit (Fig. 4B, ROD10FL and ROD14FL). The calculated gp36/gp140 ratios, represented graphically in Fig. 4B, further demonstrate that the active ROD10 and inactive ROD14 envelope proteins have indistinguishable cleavage efficiencies. These data suggest that although precursor cleavage is required for activation of the ROD Env viral release activity, it does not account for the lack of activity of the ROD14 envelope glycoprotein.

The ROD14 envelope has a trans-dominant negative effect on viral particle release

Another possibility to account for the lack of activity of the ROD14 Env may involve subtle changes in the envelope's tertiary structure. Indeed, it has been shown that the equivalent HIV-1 protein, Vpu, requires the proper formation of higher order homooligomeric structures to exert its positive effect on viral particle release (Schubert et al., 1996b). In addition, we found that expression of CD4 at the cell surface efficiently blocked the virus-release activity of Vpu, presumably through the formation of inactive heterooligomeric complexes between CD4 and Vpu (Bour et al., 1999a). Accordingly, the ability or inability of HIV-2 envelope molecules to facilitate virus release may depend on their ability to form proper oligomers structures. We addressed this possibility by testing whether coexpression of the inactive ROD14 envelope with the active ROD10 envelope could interfere with the activity displayed by the ROD10 envelope protein. For that purpose, HeLa cells were cotransfected with the Env-defective pROD10.env1 or wild-type pROD10 plasmid DNA and CMV-driven vectors expressing either the ROD10 (pCM10) or the ROD14 (pCM14) Env proteins. An envelope-deficient mutant of pCM10 (pCM10.env1) was used as a control. Cells were pulse-labeled for 30 min with [^{35}S]methionine and the particle release efficiency was assessed by pulse-chase analysis. Labeled Env products from the cell fractions were immunoprecipitated and separated on SDS–PAGE. As shown in Fig. 5A, the ROD10 and ROD14 envelope proteins were efficiently expressed in trans from their respective CMV-driven vectors; no major differences in their relative expression levels were observed. The presence of the ROD10 or ROD14 Env in trans did not lead to detectable changes in the synthesis or rate of maturation of Gag protein, whether in the context of ROD10.env1 or wild-type ROD10 (data not shown). Gag proteins immunoprecipitated at each time point of the chase from both the cell and the

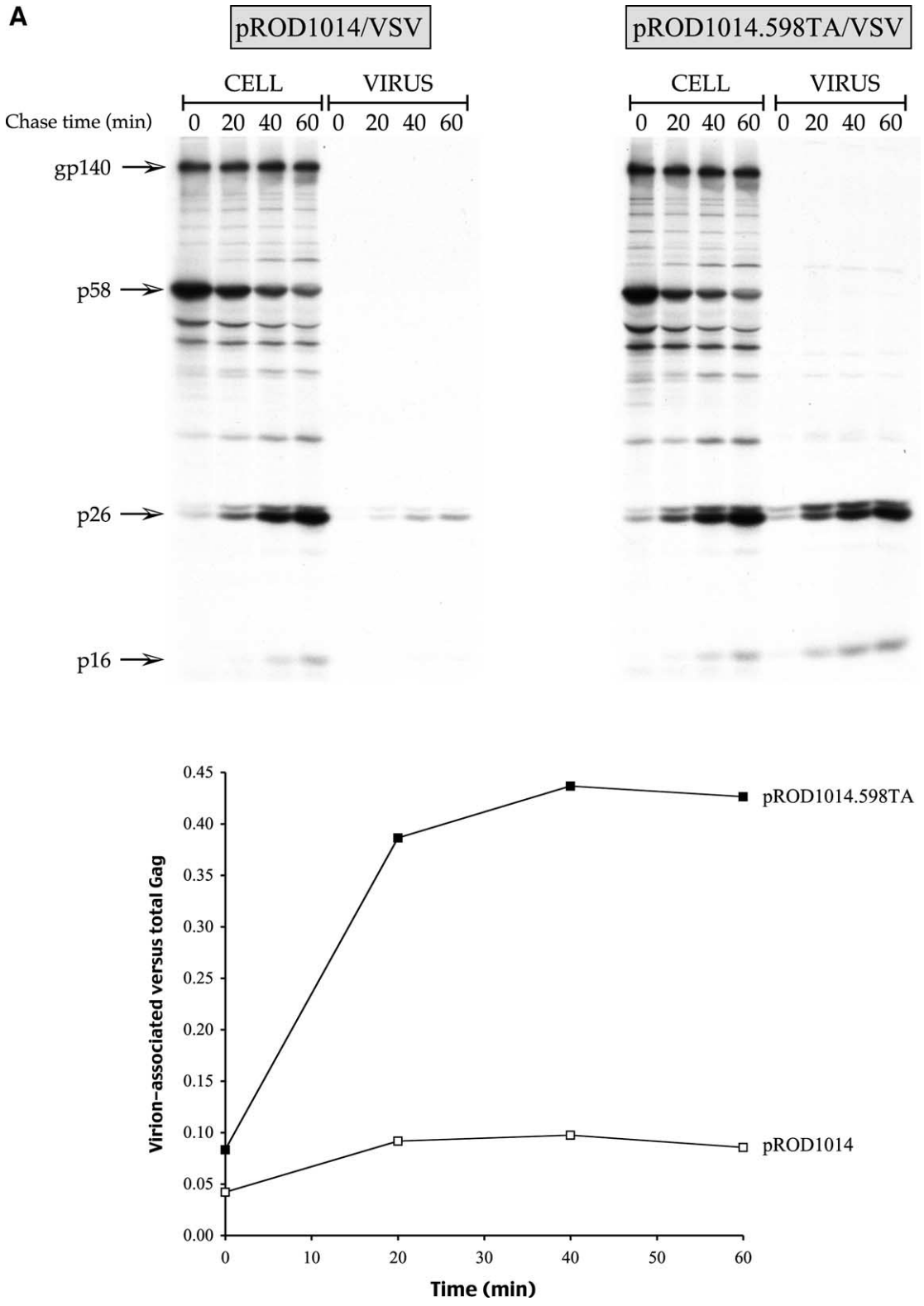

Fig. 3. Cell-type dependence of HIV-2 ROD particle release efficiency. (A) VSV-G-pseudotyped preparations of ROD1014 and ROD1014.598TA were used to infect HeLa cells. Twenty-four hours postinfection, cells were pulse-labeled for 30 min with Trans^{35}S-Label and chased for the indicated times. Panel 1: immunoprecipitation and detection of viral proteins is as described for Fig. 1. Panel 2: bands corresponding to the major HIV-2 Gag proteins in panel 1 were quantified with an image analyzer and the efficiency of particle release at each time point was calculated as described for Fig. 1. (B) Pseudotyped HIV-2 virion preparations were used to infect 12D7 and H9 cells. Cells were infected for 5 h at 37°C with equal reverse-transcriptase units of the viral species indicated. The kinetics of de novo virus production was assessed by monitoring the reverse transcriptase activity released in the culture medium over time.

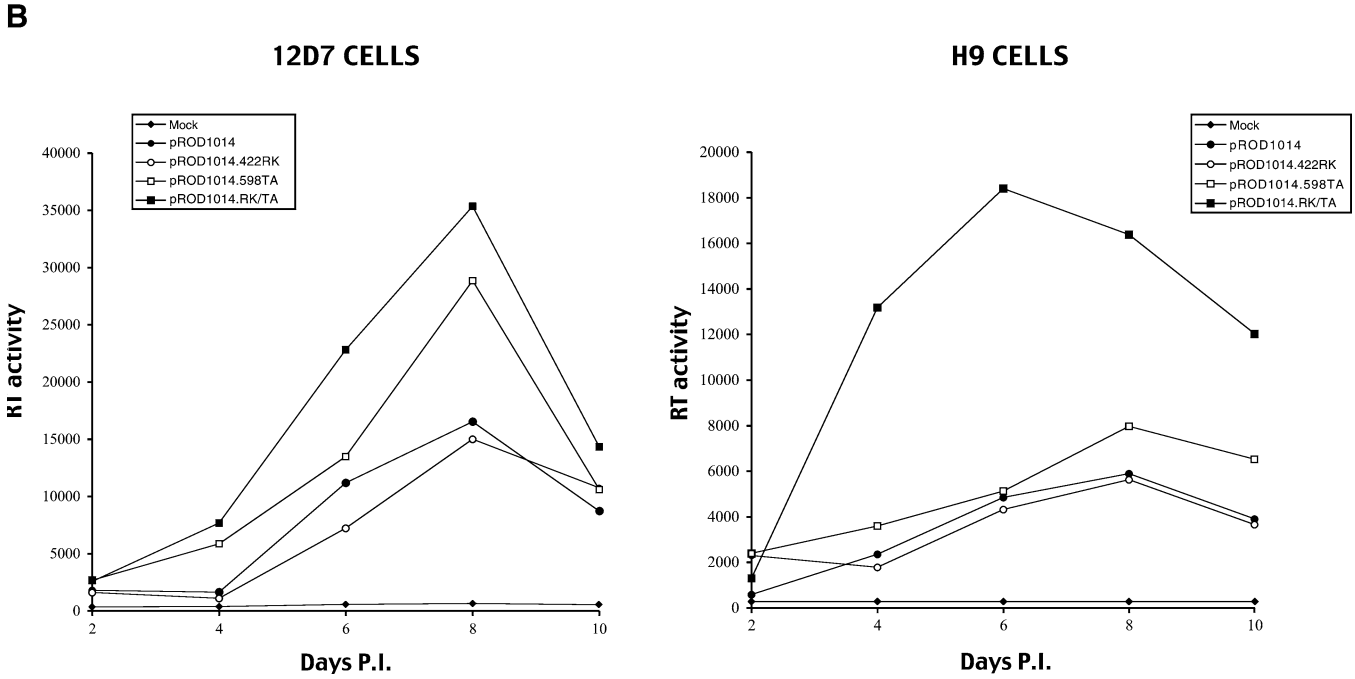

Fig. 3 (*continued*)

supernatant fractions were quantified as described above and particle release efficiency was plotted graphically in Fig. 5B. As described previously, the Env-defective ROD10.env1 had a low particle release efficiency (Bour et al., 1996). Coexpression of the ROD10 Env led to a twofold enhancement of particle production, in agreement with previous studies (Bour et al., 1996). Interestingly, expression of the ROD14 envelope protein in trans did not inhibit the particle release efficiency of the Env-defective ROD10.env1. Indeed, the particle release efficiency of ROD10.env1 was identical whether expressed in the complete absence of Env proteins (Fig. 5B, ROD10.env1 + pCM10.env1) or in the presence of the ROD14 Env (ROD10.env1 + pCM14). These data indicate that the low particle release efficiency of the ROD14 virus is due to a lack of activity on viral production rather than a possible inhibitory activity of the ROD14 Env. In agreement with our previous data (Bour et al., 1996), the particle release efficiency of ROD10 in the presence of the empty CMV-expression vector was high (Fig. 5B, ROD10 + pCM10.env1) and was unaffected by the coexpression of the ROD10 envelope in trans (Fig. 5B, ROD10 + pCM10). In contrast, coexpression of the ROD14 envelope protein had a pronounced inhibitory effect resulting in a twofold drop in the ROD10 particle release efficiency (Fig. 5B, ROD10 + pCM14). In fact, the efficiency of ROD10 virus release in the presence of the ROD14 envelope was very similar to that observed for the envelope-deficient ROD10.env1 mutant (see Fig. 1B), suggesting a complete inhibition of the ROD10 Env virus-release activity by the ROD14 Env protein. This *trans*-dominant negative activity of the ROD14 envelope on ROD10 but not ROD10.env1 strongly suggests that the virus release activity of ROD10 Env depends on the formation of proper homooligomeric structures. By intercalating itself into functional ROD10 Env oligomers, the ROD14 Env might be able to perturb the structure of the ROD10 multimer and inactivate its particle release function. The ability of a given Env protein to form such structures is therefore likely the determining factor in activating its viral particle release activity.

Discussion

Regulation of virus release is a classical example of how different solutions to common problems can evolve even in closely related viruses. While HIV-1 uses a unique accessory protein, Vpu, to facilitate virus release, a similar activity is controlled by the envelope glycoprotein of some HIV-2 isolates, including ROD10 and HIV-2 ST, as well as some SIV isolates (Bour et al., 1996; Bour and Strebel, 1996; Ritter et al., 1996). Interestingly, even though both the HIV-2 ROD10 and the ST isolate encode envelope proteins with Vpu-like activities, the structural domains within Env responsible for these activities appear to differ. In the case of the ST isolate, this biological activity is regulated by the length of the envelope's cytoplasmic tail. Indeed, the ST2 variant, which envelope is truncated to 17 cytoplasmic residues, has lost the ability to enhance virus release (Ritter et al., 1996). In contrast, the particle release activity of the ROD10 envelope is not regulated by the length of its cytoplasmic tail (Bour et al., 1999b) but, as shown in this study, requires sequences in the ectodomain of the TM subunit. Despite these differences in the structural

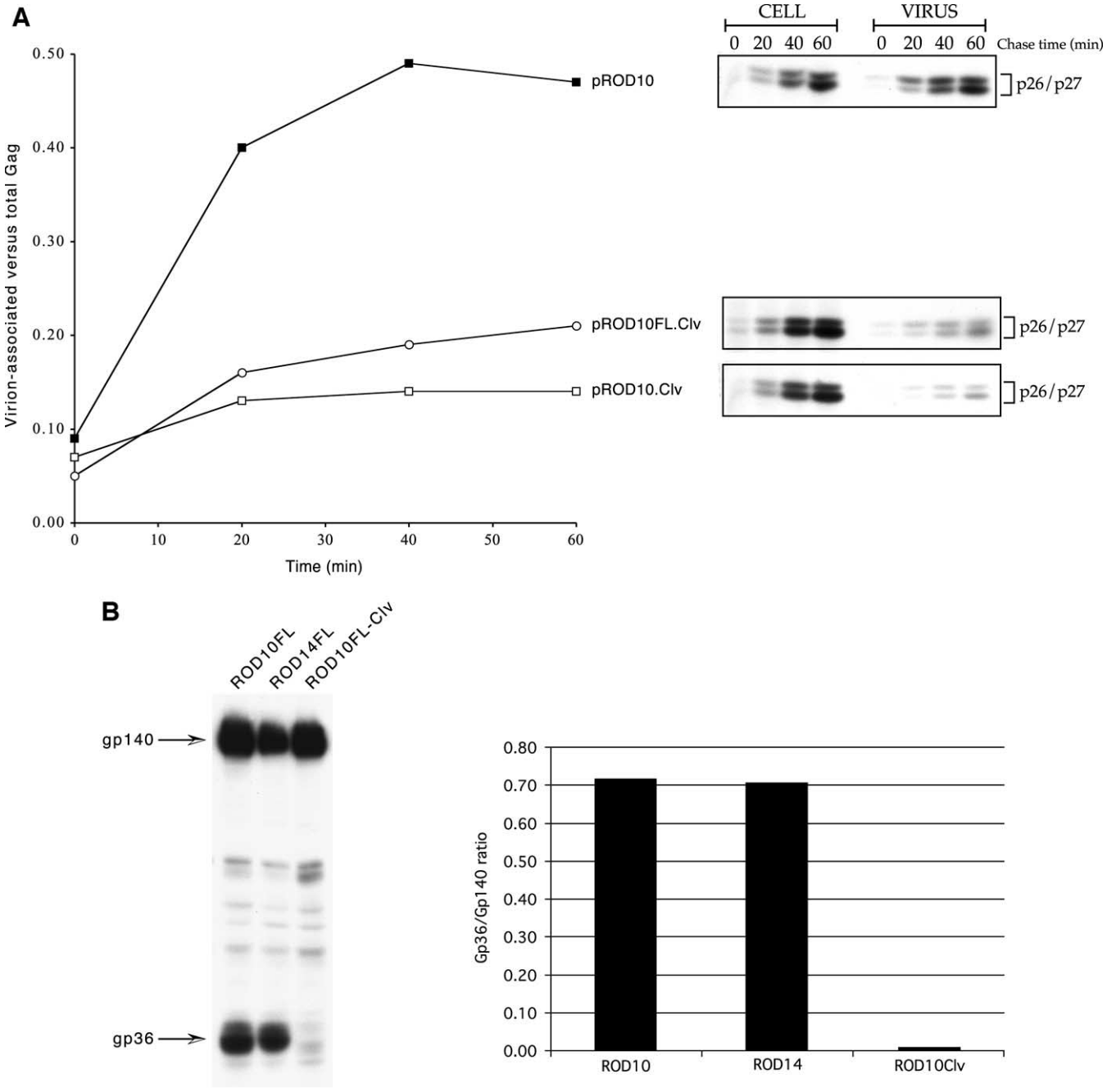

Fig. 4. Effect of ROD Env cleavage efficiency on particle release activity. (A) HeLa cells were transfected with plasmid DNA encoding the indicated HIV-2 species and subjected to pulse-chase analysis as described above. Viral capsid proteins recovered in the cell and virus fractions at each time point are shown on the right. Bands corresponding to the major HIV-2 Gag proteins ($p58^{gag}$, $p26–27^{CA}$, and $p16^{MA}$) were quantified and the particle release efficiency was calculated and plotted as a function of time. (B) HeLa cells were transfected with plasmids pROD-A2.flg (ROD10FL), pROD-A14FL.flg (ROD14FL), and pROD-A2clv.flg (ROD10FL-Clv). Cells were lysed in detergent buffer and equal amounts of the lysates were separated by SDS–PAGE. The FLAG epitope-tagged gp140 Env precursor and gp36 mature TM subunit were detected by Western blotting using the M2 anti-FLAG antibody. Proteins were quantified and the cleavage efficiency was calculated and plotted as the ratio of gp36/gp140 for each envelope protein species.

requirements, it is plausible that regulation of virus release in HIV-1, HIV-2, and SIV is based on the same molecular mechanism involving homooligomerization of the effector protein.

Our biochemical characterization suggests that the particle release activity of the HIV-2 ROD envelope protein can be regulated by a single amino acid change at position 598 in the gp36 ectodomain. This conclusion is based on the characterization of the envelope protein of ROD14, an HIV-2 infectious molecular clone closely related to ROD10

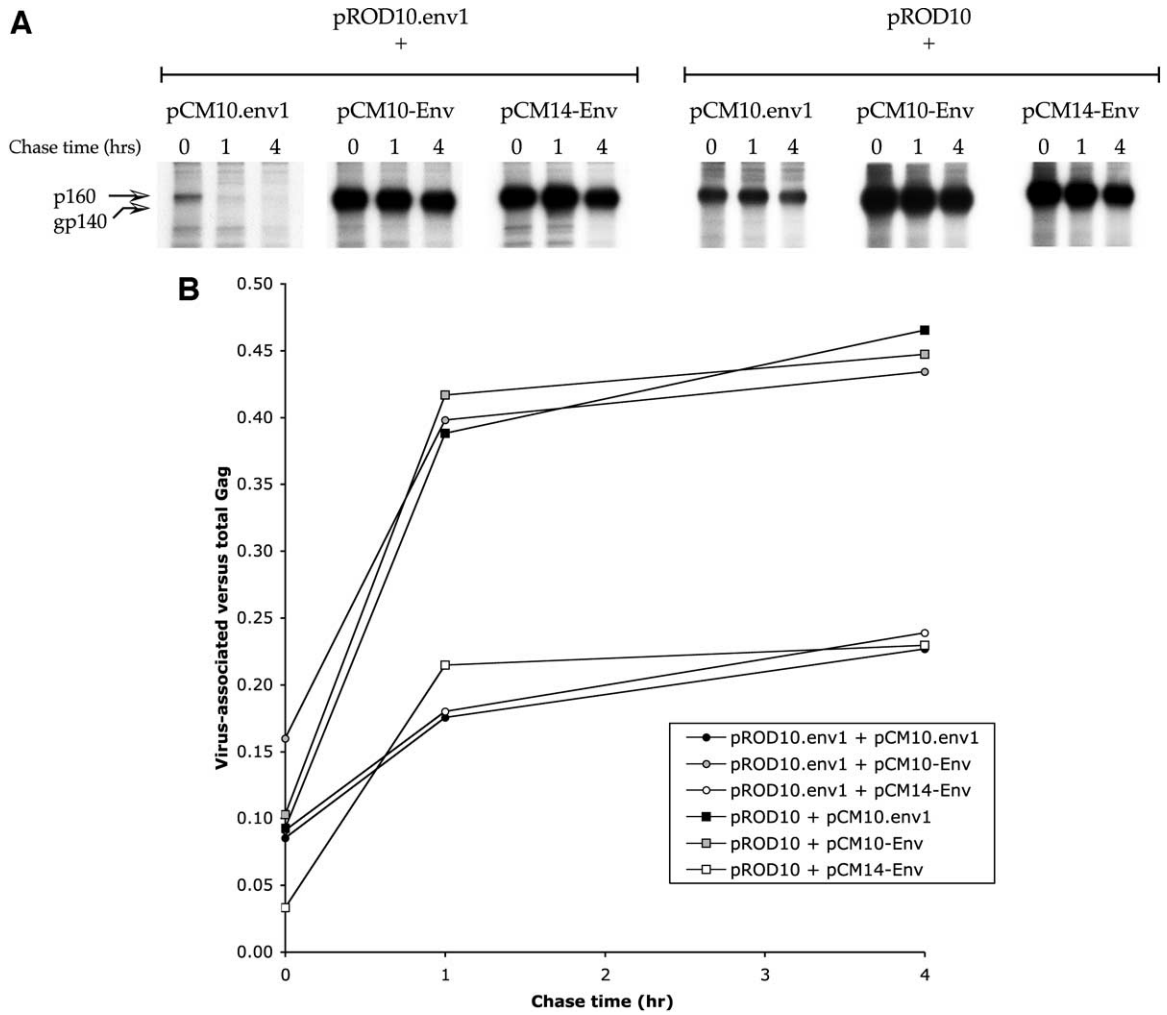

Fig. 5. Negative *trans*-dominant activity of the ROD14 Env on HIV-2 ROD10 particle release. (A) HeLa cells were transfected with 20 μg of the pROD10.env1 or pROD10 plasmid in the presence of 10 μg of either the Env-deficient plasmid pCM10.env1, the ROD10 Env-expressing vector pCM10, or the ROD14 Env-expressing plasmid pCM14. Expression of the various HIV-2 Env proteins is shown after immunoprecipitation of the cell fraction at each time point. (B) Viral particle release of cells transfected in (A) was assessed by pulse-chase analysis and immunoprecipitation. HIV-2 Gag proteins were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legend to Fig. 1 and the ratio of virion-associated versus total Gag proteins was plotted as a function of time.

(Ryan-Graham and Peden, 1995). Pulse-chase experiments performed in HeLa cells revealed that the envelope protein of ROD14 was unable to promote viral release but could be made functional by changing the threonine at position 598 to the alanine present in ROD10 Env. This mutation restored full particle release activity in the ROD14 Env without generating a broad modification of the envelope's biochemical properties such as synthesis, stability, transport, cleavage, surface expression, and virion incorporation. In HeLa cells, the 598TA mutation was necessary and sufficient to modulate particle release activity. However, studies in the CD4-positive H9 and 12D7 T cell lines also revealed a role for the residue at position 422. Indeed, only in the presence of the 422RK mutation did the 598TA substitution lead to enhanced particle production in H9 cells. The residue at position 422 is located in the C4 domain of the SU subunit, a domain known to mediate CD4 binding, fusion activity, and viral tropism (Keller et al., 1993; Otteken et al., 1993), while the residue at position 598 is located within a highly conserved region of the HIV-2 TM subunit (Korber et al., 1997) and is an alanine in most HIV-2 and SIV isolates, including ROD10. Only one naturally occurring isolate other than ROD14 has a threonine at position 598: HIV-2 UC2 (Barnett et al., 1996). Interestingly, this isolate also bears the ROD14 arginine at position 422 in the C4 domain of the SU subunit (Barnett et al., 1996). It is therefore tempting to speculate that the coselection of specific residues at positions 422 and 598 is biologically significant and correlates with efficient virus replication in some cell types. Nevertheless, residue 422 is not as conserved as residue 598 (Kuiken et al., 2000) and it is possible that the phenotype associated with the change at position 422 (i.e., increased envelope fusion activity) is not as critical for virus replication or might be accomplished through mutations in

other regions of the envelope protein. We did not observe the emergence of revertants in prolonged cultures of H9 cells infected with the particle-release defective ROD1014 and ROD1014.422RK. In addition, sequencing of viral genomes obtained from these cultures over time did not show substitutions at positions 422 or 598. In fact, the entire env sequence was remarkably stable over time. These data are not surprising in light of the fact that, similar to the situation observed for the HIV-1 Vpu protein, the presence of an envelope protein active in promoting particle release is not essential for HIV-2 ROD replication in tissue culture systems (Strebel et al., 1988). As with Vpu, the full effect of particle release-promoting activities might only be seen in vivo where more complex selection pressures are exerted on the virus. In this regard, a number of animal studies have recently demonstrated a role for Vpu in disease progression and maintenance of high viral loads as well as reversion of Vpu-defective viruses (McCormick-Davis et al., 1998; Stephens et al., 2002).

Our attempts to decipher the molecular mechanism governing the HIV-2 Env-facilitated viral particle release did not reveal any tangible differences in the biochemical properties of the inactive (ROD14) and the active (ROD14.598TA) envelope species. These results are consistent with a model in which this biological activity of the HIV-2 envelope can be switched on or off without affecting its other essential functions. Mechanistically, we have demonstrated a need for proper cleavage of the gp140 Env precursor into mature SU and TM to activate the particle release function. Since we previously demonstrated a lack of activity of the SU subunit alone (Bour and Strebel, 1996), these data indicate that the mature TM subunit, containing residue 598, harbors the functional domain for enhanced viral release. These findings also suggest a possible explanation for the interdependence of residues 422 and 598. Indeed, it is conceivable that residue 422, located in the SU, is involved in interactions with domains of the mature TM subunit (and possibly residue 598 directly) following cleavage of the gp140 precursor. Changes in residues 422 and 598 could therefore affect the rate of shedding of the SU subunit and may be critical for the activation of the particle release activity catalyzed by the TM subunit.

An important clue concerning the possible mechanism of action of the HIV-2 Env is our finding that the inactive ROD14 had a *trans*-dominant negative effect on the active ROD10 envelope. This *trans*-dominant activity is indicative of direct protein–protein interactions between the two envelope species, most likely in the form of heterooligomers. The tertiary structure of the envelope may thus be critical to activate a particle release-promoting activity. Such tertiary structure, presumably formed by trimers or tetramers of the envelope, could be functionally equivalent to the oligomeric structures formed by the HIV-1 Vpu protein (Maldarelli et al., 1993). In the case of Vpu, oligomerization is critical for the formation of ion channels believed to mediate its particle release activity (Ewart et al., 1996; Grice et al., 1997; Schubert et al., 1996b). Although we do not have direct evidence that the ROD10 envelope forms ion channels, oligomers of the HIV-1 envelope have been shown to form membrane pores (Arroyo et al., 1995). This finding could extend to HIV-2 since both envelopes oligomerize through common domains (Center et al., 1997; Doms et al., 1990). Alternatively, the 598TA mutation might change the efficiency and/or location of the disulfide bond being formed between residues 597 and 603 (Fig. 1A). This in turn could modify the conformation of the TM subunit in a way that influences its biological activity. Further characterization of the HIV-2 ROD Env particle release activity is therefore needed to not only better understand the mechanism of Env-mediated regulation of virus release but, in addition, to gain insights into the process of retroviral particle formation and release.

Materials and methods

Recombinant plasmid DNA

Construction of the pROD10 and pROD14 molecular clones of HIV-2 has been described elsewhere (Bour et al., 1996; Ryan-Graham and Peden, 1995).

The pROD-A1 plasmid and its envelope-deficient variant pROD-A1.env1 are derivatives of pROD10 that lack the *gag* and *pol* genes but express all other viral genes (Bour et al., 1996).

pCM10 expresses the ROD10 envelope protein under the transcriptional control of the cytomegalovirus (CMV) early promoter. This vector is similar to the previously described pCM5-Env (Bour and Strebel, 1996) from which it differs by the absence of an *Nco*I site in the CMV promoter. Plasmid pCM14 is similar to pCM10 but encodes the ROD14 envelope. The construct was generated by cloning the PCR-amplified full-length ROD14 envelope sequence flanked by *Xba*I and *Bam*HI restriction sites into the corresponding sites in pCM10.

pROD1014 is a derivative of the ROD10 molecular clone bearing the envelope gene from ROD14. This plasmid was constructed by cloning a 2397-bp PCR-amplified *Bsa*AI-*Bsm*I fragment from pROD14 into the corresponding sites in pROD10. The same insert was cloned into pROD-A1 to give rise to pROD-A14.

Site-directed mutagenesis

Site-directed mutagenesis of the ROD10 and ROD14 envelopes was performed using the Altered Sites Mutagenesis System (Promega, Madison, WI). Plasmids pALT-1 and pALT-11 served as templates for mutagenesis of the envelope cytoplasmic tail. The plasmids were constructed by cloning a 793-bp *Nco*I fragment (Env residues 608–858) from pROD10 and pROD14, respectively, into pALTER.Ex1 (Promega). The mutations were introduced into the pROD10 or pROD1014 molecular clones as an *Nco*I

fragment. pROD10.FL (720ZQ, a full-length 157-residues ROD10 envelope) was constructed by substituting a CAG for a TAG codon at position 2158. Plasmid pALT-17 served as template for the introduction of the 422RK, 598TA, and 422RK/598TA mutations. The vector was constructed by cloning a 1143-bp *Pst*I fragment from pROD14 (Env residues 391–772) into pALTER.Ex1. Mutant envelopes were transferred as a *Pst*I fragment into pCM14 and subsequently from the pCM14 mutants into pROD1014 as a *Pma*CI-*Bsm*I fragment and into pROD-A14 as a *Pml*I-*Bsm*I fragment.

Plasmid pALT-14 results from the mutagenesis of pALT-1 that removed the premature stop codon in the cytoplasmic tail of the ROD10 envelope. pALT-14 was used in turn as mutagenesis template to generate a C-terminal FLAG epitope-tagged (Eastman Kodak, Rochester NY, USA) version of the full-length ROD envelope proteins. An 817-bp *Nco*I containing the fused FLAG epitope was subsequently transferred from pALT-14 into pROD-A1 and pROD-A14 to generate pROD-A2.flg and pROD-A14FL.flg, respectively.

Plasmid pALT-24 was constructed by cloning a 1143-bp *Pst*I fragment from pROD10 (Env residues 391–772) into pALTER.Ex1. Plasmid pALT-25 results from the mutagenesis of pALT-24 to introduce a 511RT mutation that inactivates the ROD10 envelope cleavage site as previously described (Freed and Myers, 1992). The 511RT mutation was transferred from pALT-25 into pROD10 as described above to give rise to pROD10.Clv. A full-length envelope version of the cleavage mutant (pROD10FL.Clv) was obtained by transferring a 793-bp *Nco*I fragment from pROD10.FL into pROD10.Clv. The 511RT mutation was carried into pROD-A2.flg as a 2787-bp *Bam*HI-*Hin*dIII fragment to give rise to pROD-A2Clv.flg.

Cells, infection, and transfection

HeLa cells (ATCC CCL2) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). H9 and 12D7 cells were propagated in RPMI 1640 containing 10% FBS.

For transfection, HeLa cells were grown to near confluence in 25 cm^2 flasks (5×10^6 cells per flask). The medium was replaced prior to transfection and calcium phosphate precipitated plasmid DNA (25–30 μg) was added to the cells. After 4 h incubation at 37°C, the cells were subjected to a glycerol shock for 2.5 min. The cultures were then washed once with PBS and maintained in 5 ml DMEM/FBS for 16 h.

Infections were initiated in H9 or 12D7 cells by infection with VSV-G-pseudotyped viral particles. VSV-G-pseudotyped virus preparations were obtained as follows: semiconfluent 293T cells in T75 flasks were contransfected with 20 μg proviral pROD1014 plasmid DNA or its mutants and 2 μg pCMV-G expressing the VSV-G protein using the Lipofectamine Plus reagents (Life Technology). Forty-eight hours later, the culture supernatants were harvested, clarified by centrifugation, and filtrated through 0.45-mm Sterivex-HX filter (Millipore), followed by ultracentrifugation at 25,000 rpm for 1 h in an SW41 rotor. The virus pellets were resuspended in 1 ml RPMI-1640 supplemented with 10% FCS. Cells were infected at 3×10^6 cells/ml with 300,000 cpm RT activity of concentrated VSV-G pseudotyped virus for 4 h at 37°C. Fresh medium was added to 5 ml final and the infection kinetics were monitored by reverse transcriptase assay. The cultures were also monitored for cytopathic effects and formation of syncytia by examination under the light microscope.

Antisera and antibodies

Serum from an asymptomatic HIV-1 seropositive patient (TP serum) supplemented with a pool of HIV-2 patient sera and a rabbit antiserum to HIV-2_{ST} gp120 (contributed to the AIDS Research and Reference Reagent Program by Drs. Saladin Osmanov and Raymond Sweet, respectively) were used to detect HIV-2 Gag and envelope proteins.

A peroxidase-conjugated monoclonal anti-FLAG antibody (M2, Sigma Chemical, St. Louis, MO) was used for Western blot detection of the C-terminal flagged ROD10 and ROD14 envelope proteins.

Pulse-chase and immunoprecipitation

Viral particle release was assessed by pulse-chase analysis and immunoprecipitation of viral proteins was secreted into the culture supernatant in the form of pelletable virions as recently described (Bour et al., 1996; Bour and Strebel, 1996). Briefly, transfected HeLa cells were pulse-labeled with 1 mCi/ml Trans^{35}S-Label (ICN Biomedical, Inc., Costa Mesa, CA, USA) for 30 min. Cells were subjected to a chase at 37°C in 300 μl of prewarmed DMEM/FBS for the indicated chase periods. At each time point, cells were collected and lysed in 400 μl of NP-40-DOC buffer [20 mM Tris–HCl pH 8, 120 mM NaCl, 2 mM EDTA, 0.5% DOC, 1% NP-40]. The culture supernatants were filtered through 0.45-μm cellulose acetate Spin-X centrifuge tube filters (Corning Costar Corp., Cambridge, MA) to remove residual cells and cell debris. Virus particles were then pelleted from cell-free supernatants in a refrigerated Eppendorf microcentrifuge (4°C, 90 min, 16,000 *g*). Pelleted virions were lysed in 400 μl NP-40-DOC buffer. Cell lysates were precleared with Protein A–Sepharose beads (Sigma Chemical) and immunoprecipitated with a 1:1 mixture of TP serum and HIV-2 patient serum pool. Immunoprecipitates were solubilized by boiling in sample buffer containing 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 1% glycerol, 65 mM Tris–hydrochloride (pH 6.8), and separated by SDS–PAGE using 12% gels. Gels were fixed, incubated for 20 min in Enlightning (NEN Research Products, Boston, MA), and dried. Radioactive bands were visualized by fluorography using Bio-Max MR films (Eastman Kodak).

Quantitation of the relevant bands was performed using a Fujix BAS 2000 Bio-Image Analyzer.

Western blotting

Cell lysates were prepared from transfected HeLa cells 24-h posttransfection by lysis in NP-40-DOC buffer, as described above. A Bio-Rad protein assay was performed according to manufacturer's protocol to normalize the amount of protein for separation. Five to ten micrograms of proteins per lane were separated on 10% polyacrylamide–SDS gels. Proteins were then transferred onto PVDF membranes and probed with a 1/2000 dilution of anti-flag M2 peroxidase-conjugated antibody (Sigma Chemical). Proteins were visualized by ECL (Amersham Biotech, Piscataway, NJ).

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